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TITLE: LIGHT: A Novel Immunotherapy for Primary and Metastatic Prostate Cancer

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14. ABSTRACT Over-expression of LIGHT has been show in various tumor models to induce tumor regression and tumor immunogenicity. However, the models are based on transplanted tumors that express artificial foreign antigens that function as tumor antigens, LIGHT has never been evaluated in prostate cancer, where self-antigens likely exist. We have provided the first evidence that LIGHT-induced T cells are specific for at least one relevant prostate expressed self-antigen, PSCA. We have also demonstrated that LIGHT treatment in prostate cancer has a positive effect on the tumor microenvironment, which suggests a strong likelihood that combination treatment with LIGHT and immunotherapeutic vaccination will have an impact against primary and possibly metastatic prostate cancer. Thus, therapeutic intervention by delivering LIGHT to the tumors may serve the dual purpose of inhibiting immune-suppression mediated by regulatory T cells while simultaneously activating tumor-specific immune responses, which we hope to demonstrate can be boosted by vaccination. This study may potentially provide a practical means of overcoming tumor-mediated immunosuppressive mechanisms in a variety of solid human tumors, including those of the prostate, which would have important implications for patients who are diagnosed at the later stages of disease and currently have no recourse for treatment.					
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INTRODUCTION

Prostate cancer is the second leading cause of cancer-related death in the United States and according to the American Cancer Society's most recent estimates, will affect almost 200,000 men in 2009. Of these, almost 30,000 men are estimated to die in the United States [1, 2]. Much of the focus of past and current research aims to improve methods to detect the disease at the very earliest stage of carcinogenesis. However, treatment options remain limited [3]. In many cases, expectant management or "watchful waiting" is the standard of care. The current modalities available for prostate cancer treatment have debilitating side effects which include, but are not limited to, urinary, bowel and erectile dysfunction, loss of fertility, effects due to the loss of testosterone (including fatigue, decreased sexual desire, weight gain, loss of muscle mass and osteoporosis) and the well-known devastating side effects of chemotherapy [4, 5]. Metastatic prostate cancer is a death sentence as it is infeasible to remove metastasis by radiation, surgery or any other existing modality. There is no cure for advanced prostate cancer, and thus, there is a significant need to focus research efforts on developing new therapeutic strategies.

While surgery or radiation therapy may be used to treat primary tumors, once the disease spreads beyond the prostate, immunotherapy may be the only way to treat it [6, 7]. A majority of clinical trials for the immunotherapy of prostate cancer have yielded results similar to those seen for most other cancers, which is the induction of tumor-specific immune responses yet limited success in terms of regression or survival. Despite the 2009 U.S Food and Drug Administration (FDA) approval of PROVENGE, the first immunotherapeutic cell-based vaccine that can be prescribed for hormone-refractory prostate cancer patients, excitement is dampened because there have been no objective cures [8]. The failure to clear tumors despite successful induction of immunity in the majority of clinical trials may, in part, be attributed to the suppressive environment within the tumor that disables function of the immune system. Thus, it is essential to develop therapeutic modalities that aim to generate tumor-specific immunity and simultaneously inhibit local immune suppression [9]. Since regulatory T cells appear to be central to inhibiting anti-tumor immunity, **the goal of our proposal is to establish a therapeutic intervention that can overcome the suppressive activity of regulatory T cells while simultaneously inducing prostate cancer-specific immunity.**

LIGHT, a ligand for Herpes Virus Entry Mediator (HVEM) and Lymphotoxin beta-receptor (LT β R), is predominantly expressed on activated immune cells, signaling via LT β R is required for the formation of organized lymphoid tissues while signaling via HVEM induces costimulation [10-13]. Although LIGHT has not been extensively studied in the prostate cancer setting and has not been associated with the inhibition of Treg development or function, our previous experience using LIGHT in a virally-induced tumor model suggests a strong connection between forced LIGHT expression in tumors with a survival benefit and change in tumor milieu [14-16]. Therefore, **we hypothesize that Treg formation and function within the tumor microenvironment can be inhibited by the forced expression of the costimulatory molecule, LIGHT, thereby improving the efficacy of therapeutic vaccines in the absence of a suppressive tumor microenvironment where strong anti-tumoral response may emerge, resulting in an increase survival and tumor specific immunogenicity.** Thus we have proposed the following aims: Aim 1) To determine whether forced expression of LIGHT can inhibit prostate tumor-induced differentiation and function of CD4⁺ regulatory T cells; Aim 2) To determine whether forced expression of LIGHT can alter the pattern of infiltration and maturation of immune cells, other than T cells, within the tumor microenvironment; Aim 3) To determine whether forced expression of LIGHT in combination with vaccination can induce regression of well-established primary and metastatic prostate tumors.

BODY

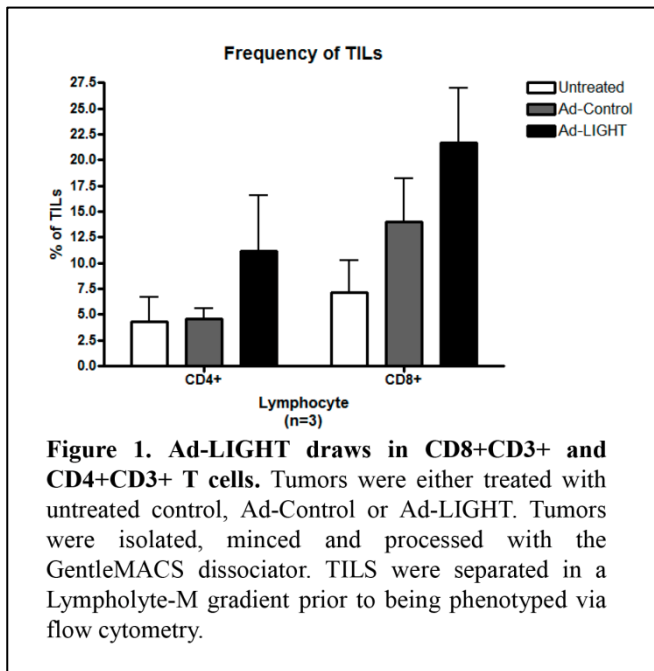
SPECIFIC AIM 1: Determine whether forced expression of LIGHT can inhibit prostate tumor-induced differentiation and function of CD4+ regulatory T cells.

Task 1.1 Compare the effect of treatment with Ad-LIGHT on frequency and function of CD4+ T cells.

Task 1.1 was completed in the last progress report.

Task 1.2 Determine whether tumors induce differentiation of naïve CD4+ T cells into Tregs

In the previous progress report, we demonstrated that the expression of LIGHT alters the tumor microenvironment by drawing in TILs with an increased ratio of T responder (CD3+) cells versus Tregs. However, we were unable to distinguish the true phenotype of the TILs due to the number of tumor cells and debris that masked the lymphoid markers. Therefore sensitivity of detecting surface markers from the minor population of TILs was obstructed. We mitigated this problem by using the GentleMACS dissociator (Miltenyi, Auburn, CA), a small benchtop instrument for the automated dissociation of tissues into single-cell suspensions, and isolating TILs from tumor debris with a sugar based gradient, Lympholyte-M.



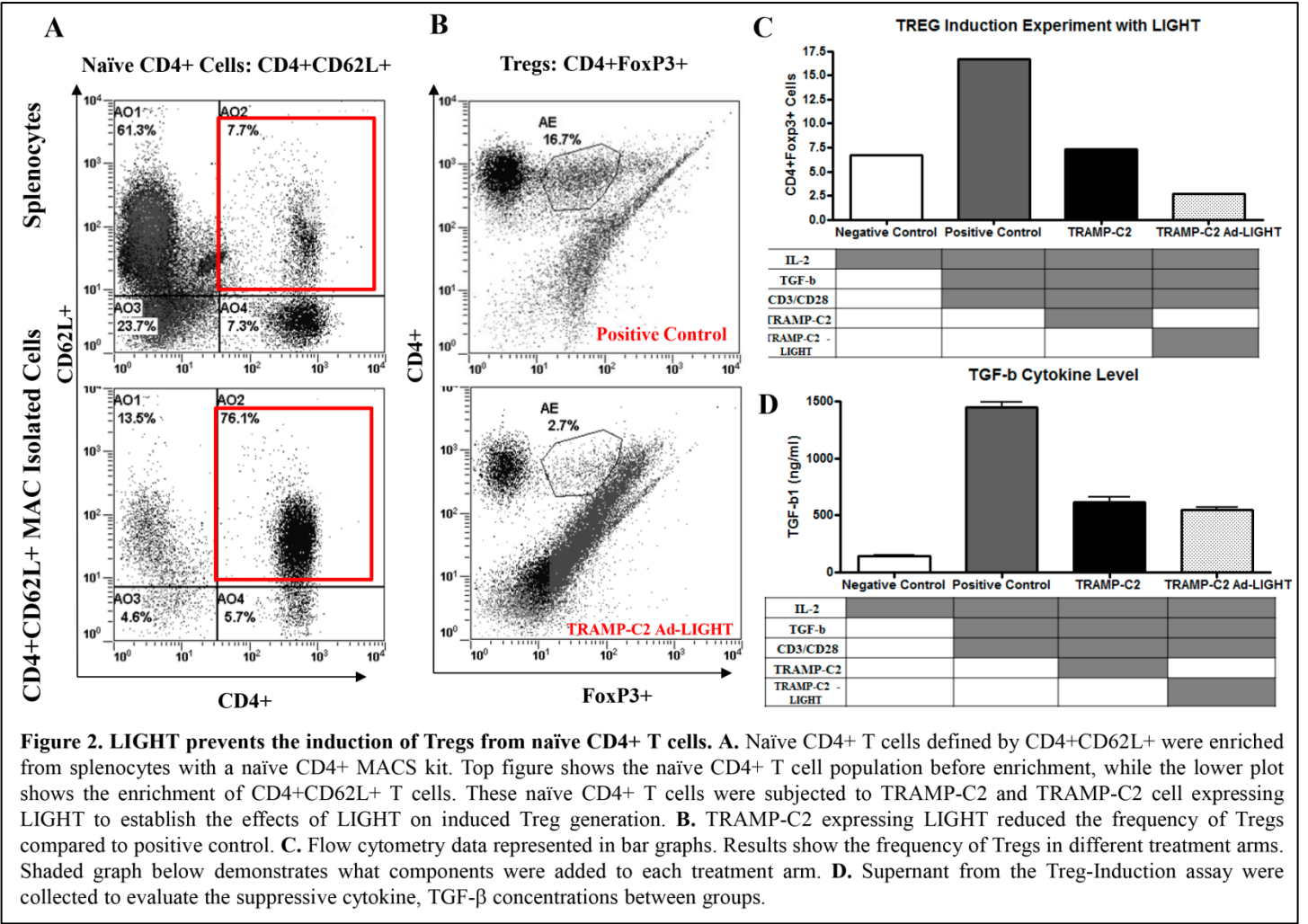
We show in **Figure 1**, that we were able to tease out the specific population of T cells that infiltrated into the tumor microenvironment with the GentleMAC and Lympholyte-M strategy. Compared to untreated control and Ad-Control population, Ad-LIGHT induced a higher frequency of infiltrating effector T cells into the tumor. Majority of the TILs detected in Ad-LIGHT treated tumors were CD8+CD3+ (21.7 %) and CD4+CD3+ (11.2 %) T cells. These results confirm and extend our previously reported results where a massive infiltration of CD3+ T cells was detected with Ad-LIGHT treatment. Additionally, the increased frequency of cytotoxic CD8+ T cells and helper CD4+ T cells supports and potentially explains our original preliminary findings, where Ad-LIGHT treatment induced mPSCA specific CD8+ T cells that resulted in a delay in tumor growth and an increase in survival. We will

continue to repeat these experiments with a higher n value to further establish significance.

Task 1.3 Determine whether forced expression of LIGHT in tumor can prevent the differentiation of naïve CD4+ T cells into Tregs.

Task 1.3 requires the breeding of TRAMP mice with *FoxP3* transgenic mice, to generate a model that will spontaneously develop prostate cancer but their regulatory T cells may be depleted via administration of diphtheria toxin. However, a major setback we encountered is that our DEREK colony (*FoxP3* transgenic mice) could not sustain itself due to lack of sufficient offspring with the correct phenotype. To remedy this we are currently obtaining new transgenic model with similar genetic background as DEREGs, known as ***FoxP3-DTR***, from Jackson Laboratory. DEREGs contain a DTR-eGFP transgene that is under the control of the *FoxP3* promoter [17], whereas *FoxP3-DTRs* contain an IRES DTR-eGFP downstream of the internal *FoxP3* stop codon[18]. The advantage of using a commercially available transgenic model rather than the DEREG model is

the availability of animals and breeder pairs. We have struggled to find other laboratories that could spare DEREK breeder pairs, but all of them are having trouble producing sufficient DEREK off spring themselves. *FoxP3-DTR* mice may be depleted of their regulatory T cells with the administration of diphtheria toxin [18]. These mice will be available for distribution in the beginning of October 2013; we have preordered them and are currently waiting for their arrival. Mice will be bred as described previously with TRAMP mice to establish a TRAMP-*FoxP3-DTR* model. Thus, we can determine whether LIGHT may prevent the differentiation of naïve CD4+ T cells into Tregs in-vivo.



Despite the current lack of *FoxP3-DTR* transgenic model, we have explored the mechanism of LIGHT and Treg induction in-vitro (see **Figure 2**). Naïve CD4+ T cells were enriched from spleens via the Milteny MACS kit for CD4+CD62L+ T cells (**Figure 2A**). We employed an in-vitro induction of Tregs with stimulants including TGF-β (5 ng/mL), IL-2 (100 U/mL) and CD3/CD28 stimulating dynabeads. Treatment groups of naïve CD4+ T cells were subjected to stimulants including TRAMP-C2 cells and TRAMP-C2 LIGHT expressing cells to examine the frequency of Tregs subsequent to LIGHT interaction. These results reflect on the ability of LIGHT to convert naïve CD4+ T cells to suppressive modulators of the tumor microenvironment. Flow cytometry was employed to phenotype Tregs (CD4+FoxP3+). Flow cytometry dot plots in **Figure 2B** are gated on the Treg population and assesses the frequency of Tregs in the Positive Control and TRAMP-C2 Ad-LIGHT treatment arms (other treatment plots are not displayed). Approximately 16.7% of the naïve CD4+ population were converted into Tregs in the positive control, whereas naïve CD4+ cells that were exposed to LIGHT on TRAMP-C2 cells induced only 2.7% of Tregs. This data was converted into a bar graph, as seen in **Figure 2C**. Here also shown are the other treatment groups including a negative control, positive control, TRAMP-C2 control, and TRAMP-C2 Ad-LIGHT. The expression of LIGHT on TRAMP-C2 cells dramatically reduces the

frequency of Tregs as compared to the positive control. Supernatants from each treatment arm were also collected and were assessed for the suppressive cytokine, TGF- β 1 (**Figure 2D**). Results demonstrate that Tregs from the positive control group were able to secrete higher levels of TGF- β 1 as compared to cells co-cultured with TRAMP-C2 Ad-LIGHT or TRAMP-C2 cells. These data suggest that LIGHT interaction directly affects the induction of Tregs from a naïve CD4⁺ T cell population but also that this is not reflected in different levels of TGF β .

Task 1.4 Determine the effect of forced expression of LIGHT on the differentiation and activation state of tumor-infiltrating CD4⁺ T cells.

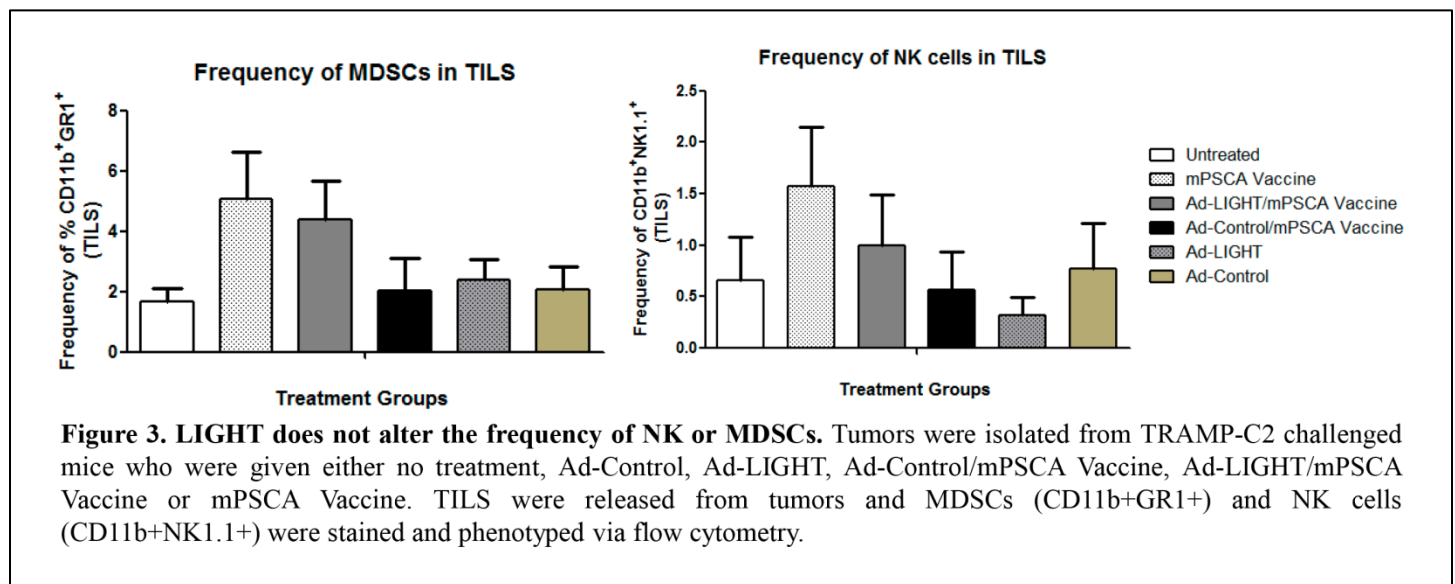
We are currently waiting for the arrival of *FoxP3*-DTR mice for this task. Once we receive the transgenic animals, we can perform an adoptive transfer of naïve CD4⁺ T cells from *FoxP3*-DTRs to TRAMP-C2 challenged mice to determine the differentiation and activation state of tumor-infiltrating CD4⁺ T cells via flow cytometry. We do not anticipate any difficulties once transgenic animals are received.

SPECIFIC AIM 2: Determine whether forced expression of LIGHT can alter the pattern of infiltration and maturation of immune cells, other than T cells, within the tumor microenvironment.

Task 2.1 Compare the intra-tumoral cytokines and chemokine profile following treatment with Ad-LIGHT

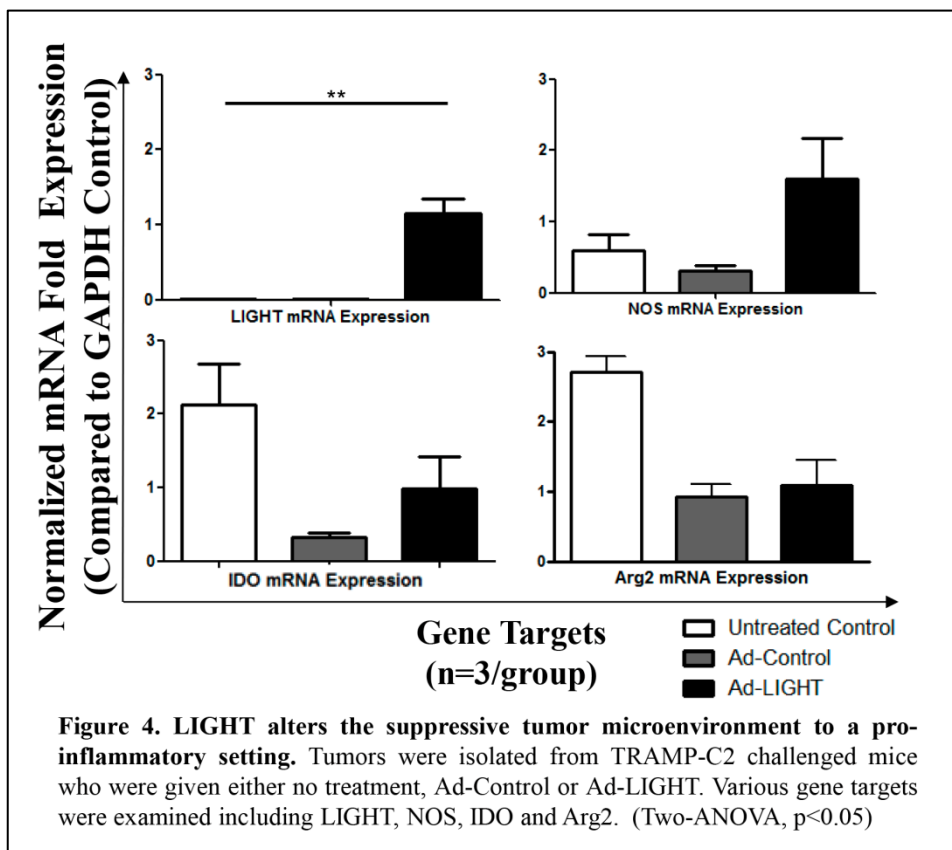
Task 2.1 was complete in the last progress report.

Task 2.2 Compare the frequency and phenotype of tumor-infiltrating cells.



We explored the frequency of NK cells and MDSCs in TILs after Ad-LIGHT treatment followed by mPSCA vaccine. The results show a trend in **Figure 3**, an increased frequency of NK cells and MDSC's are found in the tumor subsequently to mPSCA vaccination. Ad-LIGHT did not contribute to any additional infiltration of these cells. The infiltration of NK cells, also known as cytotoxic lymphocytes of the innate immune system play an important role in tumor killing and immunological control. The mPSCA vaccine shows great success in recruiting NK cells to the tumor microenvironment. However, the addition of LIGHT expression on prostate tumors does not affect theses frequencies. Interestingly, the frequency of MDSC's increased with mPSCA vaccination alone. The addition of Ad-LIGHT or Ad-LIGHT alone does not alter the frequency of MDSC's as compared to the untreated control. MDSC's are early progenitor cells that are known to have suppressive functions in inhibiting NK, CD4⁺ and CD8⁺ T cells. Additionally, MDSC's are known to induce the production

of Arginases (Arg) that inhibits T cell proliferation and induces T cell apoptosis. Since Arg and other gene targets, including nitric oxide synthase (NOS) and indolamine (IDO) [19], may be expressed by activated MDSC's, we began to explore these genes in tumors subsequent to treatment. The current data suggest that the mPSCA vaccine seems to induce favorable (NK cells) and unfavorable cells (MDSCs) while LIGHT does not affect these frequencies.



We isolated tumors from untreated control, Ad-Control and Ad-LIGHT treated mice and looked for various gene targets including LIGHT, NOS, IDO and Arg2 (**Figure 4**). As expected, LIGHT treated tumors show an increase in LIGHT expression as compared to Ad-Control and untreated control. NOS mRNA expression is elevated with Ad-LIGHT treatment. High level of NOS expression has been shown in literature to inhibit tumor growth and induce tumor apoptosis [20], whereas lower levels of NOS have been associated with promotion in tumor survival. Although LIGHT induces NOS expression, the reduction in IDO and Arg2 is not LIGHT specific but adeno-vector specific. IDO and

Arg2 mRNA expression are lower in Ad-Control and Ad-LIGHT treated tumors as compared to untreated control. These genes are known to impair T cell responses and consequently induce T cell apoptosis [21-23]. These results suggest that reduced tumor burden occurs as a result of LIGHT altering the tumor microenvironment by increasing NOS expression.

We will continue to explore other tumor infiltrating cells including Th1, Th2, Th17, NK and MDSCs after treatment to establish significance in frequency of TILs. Additionally, we will investigate gene expression of LIGHT, NOS, IDO and Arg2 in tumors that receive both Ad-LIGHT and mPSCA vaccine.

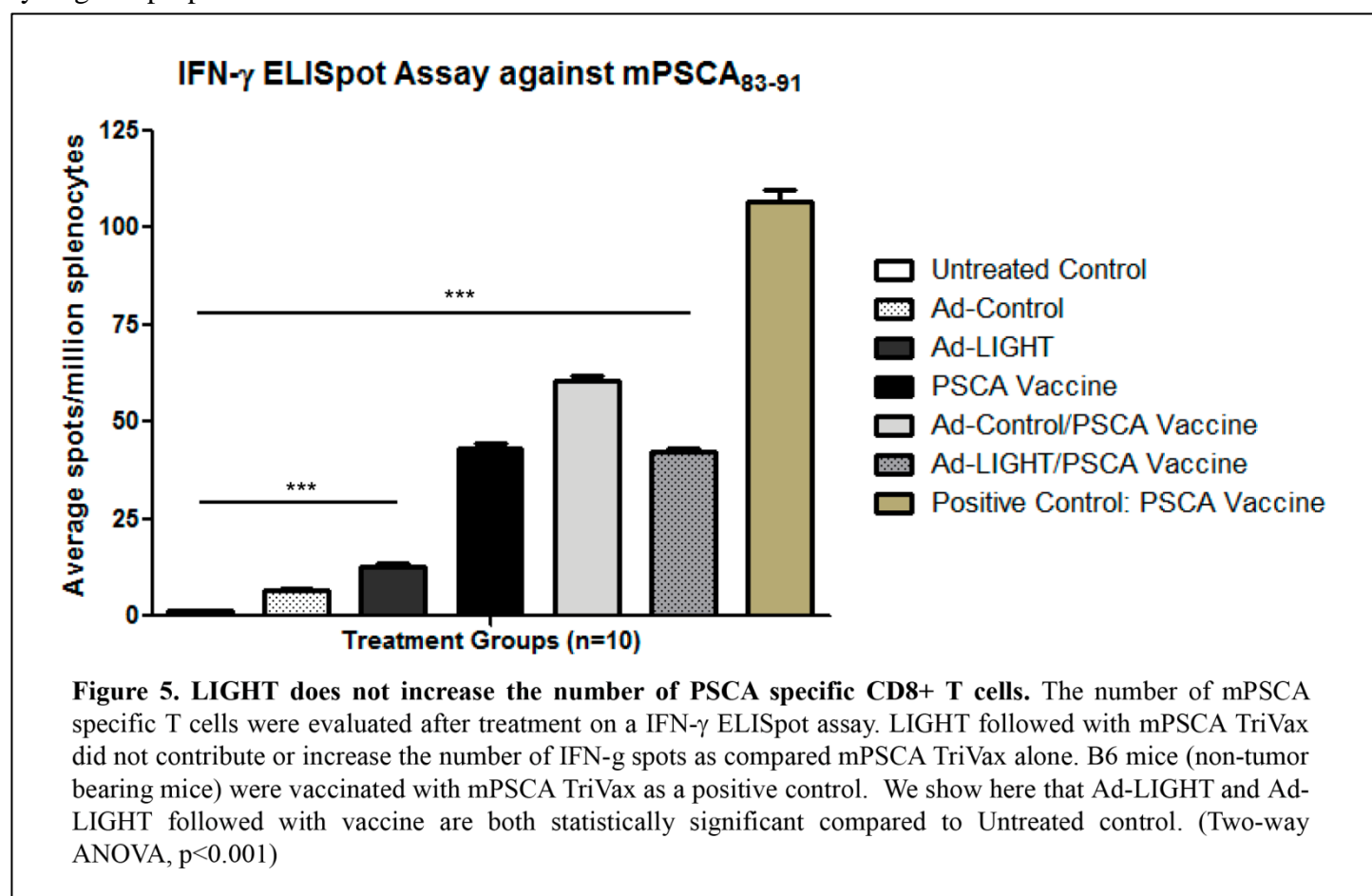
SPECIFIC AIM 3: Determine whether forced expression of LIGHT in combination with vaccination can induce regression of well-established primary and metastatic prostate tumors.

Task 3.1 Determine efficacy of treatment with Ad-LIGHT on inducing prostate cancer associated antigen-specific CD8+ T cells and regression of autochthonous primary prostate tumors in TRAMP mice.

Work on Task 3.1 will start during the current period of performance.

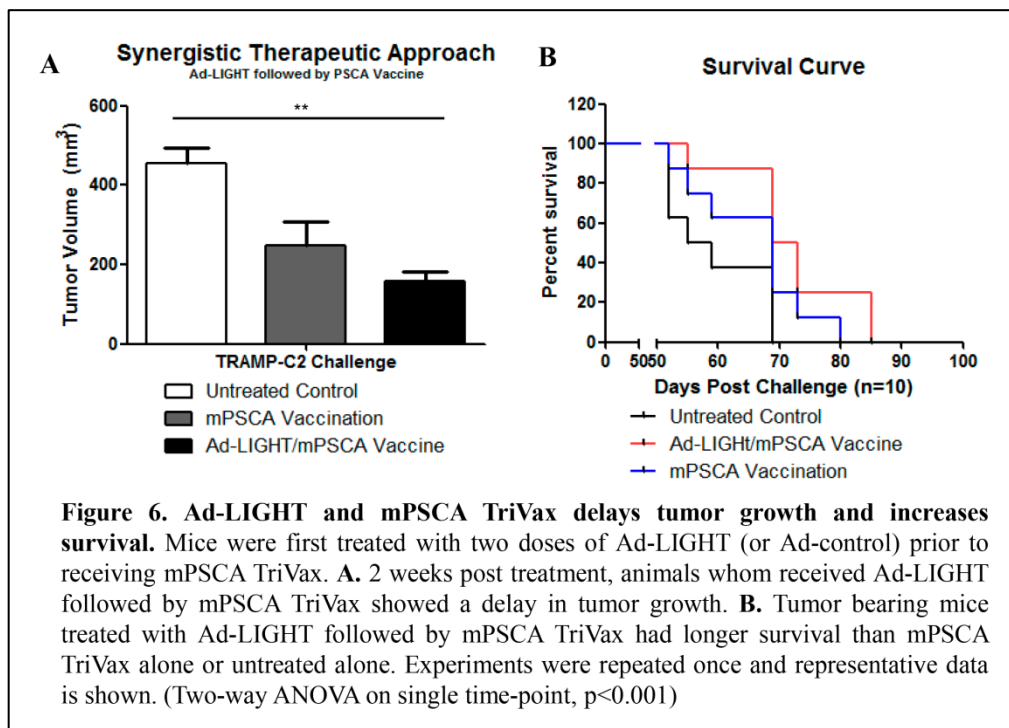
Task 3.2 Determine efficacy of treatment with Ad-LIGHT on inducing prostate cancer associated antigen-specific CD8+ T cells and regression of primary tumors in mice challenged with TRAMP-C2 cells.

We have shown in our preliminary data that LIGHT therapy is capable of inducing tumor specific immunity towards mPSCA. These results demonstrate the ability of LIGHT to induce CD8⁺ IFN γ releasing T cells, another possible mechanism that results in the delay in tumor growth and while extending survival. Building on previous results of Ad-LIGHT alone, we evaluated the synergism between Ad-LIGHT and mPSCA TriVax. Here, we show results from the IFN- γ ELISpot Assay against mPSCA (**Figure 5**) after combination treatment with Ad-LIGHT and mPSCA TriVax. We demonstrate that Ad-LIGHT alone induces an immune response towards mPSCA (as compared to untreated control). We also demonstrate that mPSCA TriVax is capable of inducing mPSCA specific T cells, yet the addition of LIGHT did not contribute to a higher influx of PSCA specific T cells. Therefore, synergism between LIGHT and mPSCA vaccine was not evident. There is a possibility that the effects of mPSCA vaccine may overshadow the effects of LIGHT despite the fact that LIGHT is capable of inducing a small population of mPSCA specific T cells. However, this experiment was performed with splenocytes, a remote organ that may not directly reflect on the true microenvironment of the tumor. Therefore, we will seek to repeat these experiments with TILS to grasp a finer understanding about the synergistic properties between LIGHT and mPSCA TriVax.



Task 3.3 Compare efficacy of treatment with Ad-LIGHT and combined treatment of Ad-LIGHT followed by PSCA vaccination in inducing regression of primary tumors in mice with TRAMP-C2 tumors.

As we have addressed in our previous report, our original collaborator, Alphavax Inc. went out of business due to lack of investor funds. Therefore, we have explored other vaccination platforms that have been shown in the field to induce an immune response towards a prostate-associated tumor antigen. We show here that mPSCA synthetic peptide vaccine, mPSCA TriVax, containing mPSCA₈₃₋₉₁, anti-CD40 antibody and Poly-ICLC, improves survival and reduces tumor burden. After evaluating this alternative vaccination platform we were able to make progress into the effect of Ad-LIGHT and PSCA therapeutic vaccination on survival.



We show in **Figure 6**, that Ad-LIGHT followed by the mPSCA TriVax reduces the tumor burden and increases survival of tumor bearing animals as compared to mPSCA TriVax alone. This synergistic approach is capable of reducing the tumor volume by more than 50% as compared to untreated control. The data suggests that Ad-LIGHT and mPSCA TriVax reduces the tumor burden and increases survival in tumor bearing animals.

Task 3.4 Determine whether combined treatment of Ad-LIGHT followed by PSCA vaccination induces

regression of metastatic tumors in mice challenged with TRAMP-C2 cells.

Work on Task 3.4 has begun but data collection will be performed during the current period of performance.

Task 3.5 Determine whether combined treatment of Ad-LIGHT followed by PSCA vaccination prevents the outgrowth of spontaneous metastatic tumors in TRAMP mice.

Work on Task 3.5 will start during the current period of performance.

KEY RESEARCH ACCOMPLISHMENTS

- Ad-LIGHT induced a higher frequency of infiltrating effector T cells into the tumor, specifically CD8+CD3+ and CD4+CD3+ T cells, as compared to the untreated and Ad-Control populations.
- LIGHT interaction directly affects the induction of Tregs from a naïve CD4+ T cell population.
- mPSCA TriVax induces infiltration of NK and MDSCs, whereas LIGHT does not affect the frequency of these cells.
- The reduction in IDO and Arg2 is not LIGHT specific but adeno-vector specific.
- Ad-LIGHT and mPSCA TriVax reduces tumor burden and increases survival in tumor bearing animals.
- LIGHT treatment may contribute to reducing tumor burden by altering the tumor microenvironment by increasing NOS expression and compromising tumor immunosuppression via Tregs.

REPORTABLE OUTCOMES

1. Oral and Poster presentation, Annual Clinical and Translational Science Meeting, May 5-7, 2013, Rochester, Minnesota.

CONCLUSION

We demonstrate the effects of LIGHT upon tumor infiltrating lymphocytes and their ability in compromising the suppressive tumor microenvironment. Forced expression of LIGHT in tumors can prevent the differentiation of naïve CD4+ T cells into Tregs. We also demonstrate that LIGHT expression increases NOS expression which may contribute to the tumor growth delay and tumor apoptosis. Our data shows the efficacy of combination treatment with LIGHT and mPSCA TriVax in reducing tumor burden and increasing survival, which suggests a future clinical impact for primary and possibly metastatic prostate cancer patients. Thus, therapeutic intervention by delivering LIGHT to the tumors may serve the dual purpose of inhibiting immune-suppression mediated by regulatory T cells while simultaneously activating tumor-specific immune responses. This study may potentially provide a practical means of overcoming tumor-mediated immunosuppressive mechanisms in a variety of solid human tumors, including those of the prostate, which would have important implications for patients who are diagnosed at the later stages of disease and currently have no recourse for treatment.

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APPENDICES

Yan, Lisa

Southern California Clinical and Translational Science Institute Oral Presentation- **May 6, 2013**

Forced LIGHT protein expression in TRAMP tumors induces prostate cancer specific immunity and increases survival.

Yan L; Da Silva D; Kanodia S; Verma B; Kast WM

Introduction: The ultimate goal of cancer immunotherapy is to stimulate the immune system to eradicate malignant tumors. One of the most common responses effective therapeutic vaccines elicit is tumor specific T cells; however, a suppressive tumor microenvironment counteracts the efficacy of these vaccines. We are in need of a therapeutic modality that will cure prostate cancer by activating immune response while eliminating tumor immunosuppression. In this study we evaluate the ability of LIGHT (a ligand for lymphotoxin beta receptor and herpes virus entry mediator) to break self-tolerance against prostate cancer antigens while eliminating immunosuppressive modulators in the tumor microenvironment. In an HPV induced cervical cancer model, forced LIGHT expression induced naïve T cells recruitment into the tumor microenvironment, HPV-specific immunity and increased overall mice survival. Forced LIGHT expression has not been studied in a prostate cancer setting where tolerance to self-antigens exists; we hypothesize that forced LIGHT expression in murine prostate cancer will increase survival by inducing prostate cancer specific T cells and inhibiting suppressive T cell functionality.

Methods: We first performed an in-vitro experiment to evaluate the ability of murine prostate cancer cells to uptake adenovirus virus encoding LIGHT (Ad-LIGHT), a method to validate our delivery system of our ligand. LIGHT expression on cell surface was determined via flow cytometry and LIGHT mRNA was evaluated via quantitative real time PCR. A subsequent series of in-vivo mouse studies were then established to evaluate the effect of LIGHT in a therapeutic cancer setting. To assess LIGHT's capacity to induce an immune response against prostate cancer, C57BL6 mice (n=10 per treatment group) were challenged with murine prostate cancer cells on day 0 and followed up to day 30 when their tumor volumes were approximately 100mm³. Tumor volumes were normalized in each group prior to treatment to void tumor volume bias. Groups were assigned to either no treatment, adenovirus vector control or adenovirus LIGHT. We then analyzed for tumor specific T cells, infiltrating T cells, functionality of suppressive T cells, tumor growth status and survival post challenge and treatment.

Results: Murine prostate cancer cells took up Ad-LIGHT and highest levels of expression were detected within the first 48 hours, 11 fold increase compared to control. Forced LIGHT drew in effector T cells into the tumor microenvironment and inhibited the production or infiltration of suppressive T cells (t-test, p<0.05). It was also shown that the suppressive T cells that were present within the system had impaired suppressive capability subsequent to LIGHT treatment. LIGHT extended mean survival (t-test, p=0.0172) and induced prostate specific T cells (Log rank test, p<0.01).

Conclusion: Forced LIGHT treatment delays prostate cancer progression in cancer bearing mice by inhibiting tumor immunosuppression and inducing prostate cancer immune specificity. We propose LIGHT as strong candidate for single-therapy treatment in prostate cancer and for future experiments with combination therapies.